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In Vivo Gene Delivery by Lentiviral Vectors

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Introduction

Successful gene therapy requires the efficient delivery and sustained expression of a therapeutic gene into the tissues of a human body. Most of the candidate tissues for therapeutic gene transfer are made of quiescent cells, such as from the brain, liver, and muscle. Thus, the optimal vector should infect nondividing cells, become stably associated with the genome of target cells, and support a high, steady-state level of transcription.[1,2] Like all vectors derived from retroviruses, lentiviral vectors integrate into the chromatin of target cells and do not transfer any viral genes. Both of these features are important for achieving sustained expression of the transgene. Moreover, lentiviral vectors infect nondividing cells, a feature sharply distinguishing them from simple or onco-retroviral vectors.[3] Upon infection, retroviruses deliver a nucleoprotein complex that reverse transcribes the viral RNA and integrates the newly made DNA into the chromatin. The nucleoprotein complexes of onco-retroviruses are excluded by the nucleus, and they reach the chromatin only when the nuclear membrane is fragmented during mitosis. This explains the dependence of a productive onco-retroviral infection on cell division occurring shortly after viral entry.[4,5] In contrast, the nucleoprotein complexes of lentiviruses contain nuclear localization signals that mediate their active transport through the nucleopores during interphase. This explains the capacity of lentiviruses to infect macrophages, a nondividing cell type.[6-9]

Lentiviruses as Potential Vectors for In Vivo Gene Delivery

Lentiviruses have a narrow range of targets limited to certain cells of the monolymphocyte lineages. In addition, lentiviruses are major pathogens for their host. Consequently, vectors derived from a lentivirus, such as HIV-1, are restricted to $CD4^+$ targets and carry the unacceptable risk of generating wild-type HIV by recombination of the constructs used to make vector.[10-13] As an alternative strategy, we generated hybrid vectors with the core derived from HIV-1, thus maintaining the ability of lentiviruses to infect nondividing cells, combined with the envelope of another virus.[14] This approach had two important advantages. First, the vector could target a broad range of tissues according to the host range of the virus donating the envelope.[15] Second, wild-type lentivirus could not be generated, as the envelope gene of the parental virus was absent from all the components used to produce vector.

Replication-defective, hybrid vectors made by the core proteins and enzymes of HIV-1 and the envelope of the vesicular stomatitis virus or the amphotropic murine leukemia virus (MLV), transduced nondividing human cells in vitro,[14,16] and neurons after direct injection in vivo.[14] A remarkable efficiency of gene transfer was achieved into the brain with long-term expression of the transgene in the absence of detectable pathology.[17] However, the biosafety of such a vector remained a crucial outstanding issue. A second important question was whether the vector could transfer genes into tissues other than the brain.

We thus embarked on the identification of the minimal genetic information required for transduction. All HIV-1 sequences found unnecessary were eliminated from the constructs used to generate the vectors. Three different constructs are used to generate vector. Two of the constructs provide the packaging functions, with one expressing the core proteins and enzymes and the other the envelope protein. The third construct expresses the transfer vector RNA. The packaging constructs express the viral proteins from heterologous promoters and polyadenylation signals and lack the *cis*-acting sequences required for the transfer of the viral genome to target cells. The transfer vector construct contains an expression cassette for the transgene(s) linked to the viral *cis*-acting sequences required for encapsidation, reverse transcription, and integration. Vector particles are assembled by the packaging constructs and only transfer the vector RNA. This limits the infection process to one round (transduction). However, recombination between the different constructs is possible during vector manufacturing and may generate replication-competent retroviruses that represent a hazard to the recipient. Using the envelope gene of an unrelated virus reduces, but does not eliminate, the risk that a recombinant acquires the sequence and originates a new type of virus.

The successful elimination of the nonessential sequences from the constructs used to generate vector resulted in a major improvement in vector biosafety. In fact, these sequences are important for the replication and pathogenesis of the parental virus, and no recombinant can acquire its full pathogenetic potential. In addition to the structural *gag*, *pol*, and *env* genes

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common to all retroviruses, lentiviruses contain two regulatory genes, *tat* and *rev*, essential for viral replication, and a set of accessory genes, *vif, vpr, vpu*, and *nef* for HIV-1, that are not required for replication in vitro but are essential virulence factors in vivo.[18,19] Producing vectors in the absence of these genes reduces, correspondingly, the risk of generating a pathogenic recombinant.

Recently, we and others showed that deletion of all four accessory genes *vif, vpr, vpu*, and *nef* from the packaging construct of an HIV-based vector did not affect the transduction efficiency of nondividing cells in culture,[20-22] and brain neurons in vivo.[20] A further gain in biosafety was achieved by the elimination of the *tat* gene. Its product is one of the most powerful transcriptional activators known and plays a pivotal role in the replication of lentiviruses.[23] We showed that the function of the *tat* gene can be provided by strong constitutive promoters upstream of the vector transcriptional start site, and that vectors generated by such constructs transduced neurons in vivo with equal efficiency, whether packaged with or without Tat.[24]

Another advantage of the elimination of HIV genes from the vector packaging constructs is that it eased the generation of stable packaging cell lines. The production of lentiviral vectors is a technical challenge because of the toxic nature of several HIV gene products. For example, Vpr causes cell cycle arrest in G_2.[25] Accordingly, vectors have been produced, until now, by the transient transfection of the required set of constructs into human kidney 293T cells. This approach, however, is hardly useful for clinical experimentation, given the difficulties in standardization and scale-up. Using inducible promoters and the more simplified packaging constructs described in the preceding paragraphs, packaging cell lines for lentiviral vectors with a satisfactory output have now been generated (L. Naldini et al, unpublished data, 1999).

Significant improvements in biosafety were also achieved by the successful generation of self-inactivating lentiviral vectors.[26,27] These vectors are produced by transfer constructs that carry an almost complete deletion in the U3 region of the HIV 3' long terminal repeat (LTR). The U3 region contains the viral enhancer and promoter, and the 3' copy is the template used to generate both LTRs of the integrated provirus. Thus, transduction of vector deleted in the 3' U3 results in the transcriptional inactivation of both LTRs. Remarkably, self-inactivating lentiviral vectors achieved a transduction efficiency and levels of transgene expression driven by an internal promoter similar to vectors carrying wild-type LTRs. We also showed that such a self-inactivating vector virtually eliminates the risk of vector mobilization and recombination with wild-type virus[28] and that, owing to the lack of interference from promoter sequences in the LTR, improves the performance of internal, tissue-specific, or regulatable promoters.[26]

The capacity of lentiviral vectors for in vivo gene delivery was first demonstrated in the central nervous system.[14,17] Rats studied over a period of nine months–the longest time tested–after a single intracranial injection of vector expressing ß-galactose (β-gal) from the cytomegalovirus (CMV) promoter showed no decrease in the average number of ß-gal positive cells and no sign of tissue pathology or immune reaction, indicating that lifelong expression of an exogenous gene can be achieved in normal animals by a single injection of vector.[29] More recently, the potential uses of lentiviral vectors for in vivo delivery were remarkably expanded by the demonstration of efficient delivery and stable gene expression in the retina, muscle, and liver.[30,31] The latter target is of particular interest for new therapeutic approaches based on gene transfer for diseases such as the deficiencies of blood clotting factors. Significant transduction of liver cells was achieved after a single intraportal injection of lentiviral vector without the need to induce liver regeneration (L. Naldini et al, unpublished data, 1999). Using this approach, therapeutically significant, sustained levels of factor IX were obtained in the serum of immunocompromised mice injected with vector carrying the human factor IX gene (L. Naldini et al, unpublished data, 1999). The dose-response and kinetics of transgene expression that can be achieved for this type of application, and the optimal design of the expression cassette in the vector are now being actively investigated.

References

1. Verma IM, Somia N. Gene therapy promises, problems and prospects. *Nature.* 1997;389:239-242.
2. Anderson WF. Human gene therapy. *Nature.* 1998;392:25-30.
3. Naldini L. Lentiviruses as gene transfer agents for delivery to non-dividing cells. *Curr Opin Biotechnol.* 1998;9:457-463.
4. Miller DG, Adam MA, Miller AD. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Mol Cell Biol.* 1990;10:4239-4242.
5. Roe T, Reynolds TC, Yu G, Brown PO. Integration of murine leukemia virus DNA depends on mitosis. *EMBO J.* 1993;12:2099-2108.
6. Lewis PF, Emerman M. Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. *J Virol.* 1994;68:510.
7. Weinberg JB, Matthews TJ, Cullen BR, Malim MH. Productive human immunodeficiency virus type 1 (HIV-1) infection of nonproliferating human monocytes. *J Exp Med.* 1991;174:1477.
8. Bukrinsky MI, Haggerty S, Dempsey MP, Sharova N, Adzhubel A, Spitz L, Lewis P, Goldfarb D, Emerman M, Stevenson M. A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. *Nature.* 1993;365:666-669.
9. Gallay P, Swingler S, Aiken C, Trono D. HIV-1 infection of nondividing cells: C-terminal tyrosine phosphorylation of the viral matrix protein is a key regulator. *Cell.* 1995;80:379-388.
10. Shimada T, Fujii H, Mitsuya A, Nienhuis W. Targeted and highly efficient gene transfer into CD4+ cells by a recombinant human immunodeficiency virus retroviral vector. *J Clin Invest.* 1991;88:1043-1047.
11. Poznansky M, Lever A, Bergeron L, Haseltine W, Sodroski J. Gene transfer into human lymphocytes by a defective human immunodeficiency virus type 1 vector. *J Virol.* 1991;65:532-536.
12. Buchschacher GLJ, Panganiban AT. Human immunodeficiency virus vectors for inducible expression of foreign genes. *J Virology.* 1992;66:2731-2739.
13. Parolin C, Dorfman T, Palu G, Gottlinger H, Sodroski J. Analysis in human immunodeficiency virus type 1 vectors of cis-acting sequences that affect gene transfer into human lymphocytes. *J Virol.* 1994;68:3888-3895.
14. Naldini L, Blömer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM, Trono D. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science.* 1996;272:263-267.
15. Burns JC, Friedmann T, Driever W, Burrascano M, Yee JK. Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and non-mammalian cells. *Proc Natl Acad Sci USA.* 1993;90:8033-8037.
16. Akkina RK, Walton RM, Chen ML, Li Q-X, Planelles V, Chen ISY. High-efficiency gene transfer into CD34+ cells with a human

immunodeficiency virus type 1-based retroviral vector pseudotyped with vesicular stomatitis virus envelope glycoprotein G. *J Virol.* 1996;70:2581-2585.
17. Naldini L, Blömer U, Gage FH, Trono D, Verma IM. Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc Natl Acad Sci USA.* 1996;93:11382-11388.
18. Luciw PA. Human immunodeficiency viruses and their replication. In: Fields BN, Knipe DM, Howley PM, Chanock RM, Melnick JL, Monath TP, Roisman B Straus SE. eds. *Fields Virology.* 3rd ed. Philadelphia: Lippincott-Raven Publisher; 1996:1881-1975.
19. Trono D. HIV accessory proteins: leading roles for the supporting cast. *Cell.* 1995;82:189-192.
20. Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nature Biotech.* 1997;15:871-875.
21. Reiser J, Harmison G, Kluepfel-Stahl S, Brady RO, Karlsson S, Schubert M. Transduction of nondividing cells pseudotyped defective high-titer HIV type 1 particles. *Proc Natl Acad Sci USA.* 1996;93:15266-15271.
22. Kim VN, Mitrophanous K, Kingsman SM, Kingsman AJ. Minimal requirement for a lentivirus vector based on human immunodeficiency virus type 1. *J Virol.* 1998;72: 811-816.
23. Wei P, Garber ME, Fang SM, Fischer WH, Jones KA. A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. *Cell.* 1998;92:451-462.
24. Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, Naldini L. A third generation lentiviral vector with a conditional packaging system. *J Virol.* 1998;72: 8463-8471.
25. Goh WC, Rogel ME, Kinsey CM, Michael SF, Fultz PN, Nowak MA, Hahn BH, Emerman M. HIV-1 Vpr increases viral expression by manipulation of the cell cycle: a mechanism for selection of Vpr in vivo. *Nat Medicine.* 1998;4:65-71.
26. Zufferey R T, Dull RJ, Mandel A, Bukovsky D, Quiroz L, Naldini L, Trono D. Self-inactivating lentiviral vector for safe and efficient in vivo gene delivery. *J Virol.* 1998;72:9873-9880
27. Miyoshi H, Blomer U, Takahashi M, Gage FH, Verma IM. Development of a self-inactivating lentivirus vector. *J Virol.* 1998;72:8150-8157.
28. Bukovsky A, Song J-P, Naldini L. Interaction of HIV-derived vectors with wild-type virus in transduced cells. JOURNAL. In press.
29. Blömer U, Naldini L, Kafri T, Trono D, Verma IM, Gage FH. Highly efficient and sustained gene transfer in adult neurons with a lentivirus vector. *J Virol.* 1997;71:6641-6649.
30. Miyoshi H., Takahashi M., Gage F.H., Verma I.M.. Stable and efficient gene transfer into the retina using an HIV-based lentiviral vertor. *Proc Natl Acad Sci USA.* 1997;94:10319-10323.
31. Kafri T, Blömer U, Peterson DA, Gage FH, Verma I.M. Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. *Nature Genet.* 1997;17:314-317.